# Introduction and thesis outline

## THE HISTORICAL CONTEXT

A long time ago, on the African continent *Homo Erectus* distinguished itself from all other species as it started to use fire in a controlled setting (1). Ironically, this milestone approximately 300 - 400.000 years ago might have been the event that triggered the evolutionary emergence of one of the most lethal infectious diseases known to man (2). Social gathering around fire in combination with smoke-induced airway damage has been hypothesized to provide the ideal environment for the emergence of *Mycobacterium tuberculosis* as a specialized human-specific pathogen causing TB (3).

*M. tuberculosis* co-evolved with mankind at every step of human evolution. The great migration of *Homo sapiens* out of Africa 100.000 years ago and its subsequent spread across the globe can be reconstructed based on the seven phylogeographical lineages of *M. tuberculosis* (4). Strains from each of these specific lineages continue to show increased transmissibility among their geographically associated human population, indicating optimal host adaptation (5).

During the Neolithic demographic transition around 10.000 years ago, agricultural advances and animal domestication gradually replaced our hunter-gatherer lifestyle, which resulted in massive population expansions. This steered *M. tuberculosis* co-evolution from a slowly progressive disease that benefits from host survival into a 'crowd disease' in which pathogen transmissibility equals evolutionary success and host survival becomes less important (4).

*M. tuberculosis* virulence increased throughout history. It burdened Egyptians around 5000 years ago (6) and plagued ancient Greeks in the form of 'phthisis' according to Hippocrates' *Of the epidemics* around 2500 year ago. It reached devastating proportions in Europe during the industrial revolution between the 18$^{th}$ and 19$^{th}$ century. Overcrowded cities, poor hygiene and smog exposure created a perfect combination for TB to thrive and no less than one in five human deaths was caused by it (7). Rich and poor alike were slowly dying of 'consumption', a mysterious disease with no cure that killed young people in the prime of their life.

The disease was viewed upon as a romantic disease that inspired artists through '*spes phthisica*', a phenomenon in which the physically wasting body inspired the creative soul and turned prosaic humans into poets (8). A famous example of this was John Keats with poems such as 'Ode to a Nightingale'. The romantic aspects of TB quickly vanished after Robert Koch identified the bacterium *M. tuberculosis* in 1882 as its causative agent.

In a relatively short period of time TB was degraded from a poet's disease to a contagious pathogen that was associated with 'the poor man's sputum'.

Despite the identification of its cause in 1882, an effective cure for TB remained to be found and treatment was limited to a combination of liver cod, sunlight and (perhaps most importantly) isolation from society in sanatoria (9). This changed from the 1950s onwards with the discovery and use of effective antibiotics. Streptomycin and para-aminosalicylic acid (PAS) were the first agents with moderate antimycobacterial efficacy (10). While promising, the first clinical report on these drugs already encountered two important aspects of TB treatment which still apply today: drug resistance and treatment side effects (10). A cure became possible with the discovery of isoniazid as TB drug (11). For over a decade, treatment with oral isoniazid and PAS for 18 to 24 months combined with intramuscular injections of streptomycin during the first 6 months became the standard TB treatment (12). The introduction of agents such as pyrazinamide in 1955, ethambutol in 1961 and rifampicin in 1966 further improved cure rates, while reducing treatment duration (12). Eventually, in 1979, a six months treatment course with the oral antibiotics isoniazid, rifampicin, pyrazinamide and ethambutol became, and still is, the standard of care in TB treatment (9, 13).

Unfortunately, the progression in TB treatment was overshadowed by an infectious disease crisis that started in the 1980's. The Human Immunodeficiency Virus (HIV) manifested itself and caused mortality on an unprecedented scale. Where TB caused approximately 20% mortality among affected individuals in Europe during the industrial revolution, HIV was accountable for over 50% of adult mortality in most African countries during the early 90's (14). HIV-induced immunodeficiency was quickly recognized to act as a TB-catalyzer and vice versa. The coinfection of the two diseases was termed 'the cursed duet' (14). An immunocompetent, latently infected individual has a 5-10% life-time risk of progressing to active TB (15). These chances increase substantially when this same individual is also infected with HIV, which is now the most important predisposing factor for the development of active TB disease (16). The increased progression and transmission of TB amongst HIV-infected individuals caused an increase in TB incidence in sub-Saharan Africa between 1990 and 2005, while stabilization or steady decrease of TB incidences was observed in countries outside Africa during this period (17).

Another impact of HIV on TB is of a more indirect nature. During the last 30 years, enormous global efforts have resulted in rapid development and implementation of anti-retroviral HIV therapy. Unfortunately, these efforts appear to have been at the cost of funds for TB treatment, as TB treatment has remained virtually unchanged compared to pre-HIV times. In 2016, TB claimed more victims than HIV and malaria combined (18).

Nevertheless, the global fund disbursed 40.4% of its funding for HIV, 29.7% for malaria and 22.4% for tuberculosis (19). In absolute terms: in 2016 a worldwide total of 19.1 billion dollar was available for HIV treatment and prevention compared to 6.3 billion for TB (18, 20). A potential explanation for this discrepancy is best embodied by the words of the director of the WHO global TB program, stating that "HIV is a disease that involves one of the most important aspects of life – sex. Tuberculosis involves the sputum of poor people, and the poor are without voice in most societies" (21).

Nowadays, TB treatment, in combination with improved sanitation, housing and nutrition, screening programs and outbreak prevention measures has resulted in a decline of TB incidence in developed countries. Nevertheless, in the developing world TB still has a profound impact and claimed an estimated 1.7 million lives in 2016 (18). This makes TB one of the top 10 causes of death worldwide and places it above road injuries. New threats are present on the horizon in the form of more extensive drug resistance and the emergence of *M. tuberculosis* genotypes with increased virulence. Combined with the current major funding gaps for TB diagnosis, treatment and research, it remains to be seen how long our 1979 drug regimen can contain this evolutionary giant.

### From the microbe's point of view: the template for success

*M. tuberculosis* is a slow-growing, rod-shaped, facultative intracellular bacterium that is primarily spread through aerosols coughed up by infected individuals. Detailed description of the evolutionary success of *M. tuberculosis* can be divided into three different categories: mycobacterial factors, host factors and treatment factors, which will be described in more detail.

### Mycobacterial factors in M. tuberculosis' evolutionary success

The unique features of *M. tuberculosis* start with the composition of its cell wall. The thick, lipid-rich combination of mycolic acids, lipomannan arabinogalactan and peptidoglycans prevents regular Gram staining and requires specific stains such as Ziehl-Neelsen (acid-fast) or auramine-rhodamine staining for identification (22). The composition of the mycobacterial wall stimulates rapid contact with innate leukocytes such as macrophages and subsequent phagocytosis (15). The unique inflammation-inducing capacity of the mycobacterial cell wall is best exemplified by complete Freund's adjuvant, a common immunopotentiator used to enhance vaccination efficacy in experimental animals, which primarily consists of inactivated mycobacteria. Upon phagocytosis, *M. tuberculosis* prevents acidification of the phagosomal compartment caused by phagolysosomal fusion (15). Subsequently, pore-forming virulence factors such as early secreted antigenic target 6 kDa (ESAT-6) enable translocation to the cytosol (23). It has been demonstrated that in the intracellular compartment, *M. tuberculosis* can prevent further degradation

and use the macrophage as a shielded niche for survival, replication and persistence (15).

Under influence of environmental stress factors such as antibiotic pressure or adaptive immunity, *M. tuberculosis* can further alter the composition of its cell wall as part of a transition into a slow-growing, non-replicating state in which it is highly resistant to host responses and antibiotics (24, 25). The ability of *M. tuberculosis* to progress to this resilient state is one of the main reasons for the long treatments, as metabolism is low and the thickened cell wall prevents entry of antibiotics into the bacterium (26). In its persistent state, *M. tuberculosis* is also able to effectively circumvent immunity and cause the clinical phenomenon termed 'latent TB' in which mycobacteria are present in the body, but do not cause active disease at that moment (15).

Mycobacterial strain variance is a virulence factor in TB pathogenesis that is gaining interest among TB researchers. Due to its slow-growing character, *M. tuberculosis* was initially viewed upon as a genetically conserved organism for which strain variation played a minor role in disease outcome (27). This assumption could be one of the reasons why the mycobacterial H37Rv strain, isolated from a patient in 1905 remains one of the most commonly used strains in preclinical TB research to date (28). Advances in genotyping technologies and clinical observations over the last two decades have proven this assumption to be false. H37Rv is deemed a laboratory strain as it is no longer isolated from patients, while strains from other genotypes have emerged at an alarming rate (29, 30). The best example of this is the Beijing genotype, identified in 1995 (31). Strains of the Beijing genotype show increased virulence and drug resistance compared to strains from other lineages, as illustrated by exceptionally high rates of drug resistance in Eurasia (32-40). Given the current high TB incidences in East-Asian countries, Beijing genotype strains are the second-most common strains responsible for TB after strains from the East-African Indian (EAI) genotype (29). Thus, an important consequence of strain variance that will also be discussed in this thesis is that the strains that currently cause the major burden of TB in patients are clearly distinct from those most frequently used for screening of novel anti-mycobacterial drugs in preclinical TB models.

**Host factors in M. tuberculosis' evolutionary success**

Worldwide, a huge human reservoir of latently infected individuals exists of which most will most likely never progress to active TB. Latent TB poses a significant challenge to the global eradication of TB as an estimated 30% of the world population can be classified as having latent TB (15). However, an immune-compromised state significantly increases the risk of TB reactivation (15). In developing countries this is best exemplified by HIV co-infection as discussed above. In the developed world, major risk factors for TB include

type 2 diabetes, alcohol use and smoking (41). Another increasing population of individuals at risk for TB reactivation comprise those receiving deliberate immunosuppression for treatment of auto-immune diseases, malignancies and organ transplants (16). A notorious example is the introduction of anti-TNF-α monoclonal antibody therapies for rheumatoid arthritis, which caused increased rates of TB reactivation in latently infected individuals (42). The association of such specific interventions like anti-TNF-α with TB reactivation does provide insight into their role in TB pathogenesis (43). Another classic example is the discovery of genetic defects as observed in Mendelian Susceptibility to Mycobacterial Disease (MSMD) (44). Patients with MSMD have genetic mutations resulting in defective IL-12 production or IFN-γ responsiveness, which renders them extremely susceptible for mycobacterial disease (44).

Anti-TNF-α treatment and MSMD highlight the importance of an intact IL-12 / T-helper 1 immune response/ IFN-γ in TB. However, this axis alone is not sufficient for an optimal host response. The current vaccine for TB, Bacillus Calmette-Guérin (BCG), induces a strong Th1 response but provides highly variable protection between 0-80% due to unknown causes (41, 45, 46). Further boosting of the Th1-inducing potential of BCG by using a modified Ankara virus did not improve efficacy (47, 48). BCG offers higher protection rates in young children. In adults, however, BCG vaccination not only has a lower efficacy for protection against TB, but might even have been a selective force contributing to the spread of virulent Beijing strains, which circumvent vaccine-mediated immunity more efficiently (41, 49). With increasing incidences of Beijing strain infections, this might even call for more selective vaccination strategies. Also, the basic principle of vaccination is that once the immune system has encountered a pathogen, it will form a more effective and efficient adaptive immune response upon re-infection. In the case of TB, it should be noted that reinfection after successful TB treatment frequently occurs and actually increases the chances of developing active TB instead of offering protective immunity (50, 51). Thus, in contrast to most other infectious diseases, survival after primary infection provides limited protection against future exposure. Combined with the variable efficacy of BCG, this indicates the complexity of TB immunology and the need for better understanding and identification of protective host responses.

Immune-compromised individuals have an increased risk of developing active TB, but the vast majority of TB patients are non-immune compromised adults, capable of inducing robust host responses (18). Thus, a final important host factor to consider is the contribution of our own immune system to disease progression. In other words: To what extent does our own immune system contribute to a detrimental course of TB? Gene expression signatures in TB have greater overlap with auto-immune diseases than with other infectious diseases (52). Also, preclinical studies show that boosting protective

T-cell-mediated IFN-γ production in TB promotes disease progression due to hyper-inflammation (53). So it appears that both immune suppression and stimulation can cause disease progression in TB. Unraveling the exact host factors and immunological mechanisms responsible is crucial for the development of host-directed therapies as possible adjunct to antibiotic treatment.

**Treatment factors in M. tuberculosis' evolutionary success**

Current strategies for global TB treatment revolve around DOTS, i.e. 'Directly Observed Treatment, Short course'. The success of DOTS depends on five distinct elements: (i) sustained political and financial commitment, (ii) diagnosis by quality-ensured microscopy services, (iii) a secured supply of high quality TB drugs, (iv) standardized recording and of course (v) Directly Observed Treatment (DOT) (18). DOT has been proven to be important to complete the 6-months treatment course successfully. TB treatment eliminates nearly all mycobacteria and most of the clinical symptoms in the first 2 months of treatment. However, longer treatment durations are required to eliminate persistent populations of mycobacteria. In these last 4 months, in which low numbers of persistent mycobacteria are treated, compliance to therapy is essential to prevent the development of drug-resistant TB. Drug resistance currently occurs in 4.1% of all new TB cases and 19% of previously treated cases (18). The impact of drug resistance in TB is substantial: treatment of drug-susceptible TB comprises a 6-months course with daily oral first line TB drugs, has a cure rate of approximately 83% and costs around 1200 dollar (18). In contrast, treatment of multi-drug resistant TB requires at least 18 months of treatment with second-line TB drugs, has a cure rate of approximately 55% and costs almost 10.000 dollar (18, 54, 55).

Probably one of the best ways to increase compliance and prevent drug resistance is to shorten treatment duration, but chemotherapeutic advancements that may shorten TB treatment have been scarce. After 40 years of silence, delamanid and bedaquiline have recently been approved as new agents for TB treatment, but remain reserved for the treatment of drug-resistant forms of TB (56, 57). Fortunately, the need for new TB treatment has been recognized and the current clinical pipeline for new TB drugs looks more promising than ever (41). Meanwhile, reducing duration of TB treatment through repurposing of other chemotherapeutic agents proved difficult. In 2014, a large phase III clinical trial to reduce treatment duration to 4 months through implementation of moxifloxacin in the multidrug regimen essentially failed (58). Although this clinical trial did not achieve treatment reduction of TB, it did provide essential information to rethink current methods and improve future drug development programs. It showed that early surrogates for treatment efficacy assessments as measured in clinical phase IIa/b trials are unreliable predictors for cure in TB (59, 60). More relevant for this thesis, it

also pointed out that current preclinical TB models require further optimization in order to increase their translational value (61).

Current research on new drugs, drug regimens and treatment duration primarily occurs in mouse TB models (62). These are readily available models that allow testing in large groups, but have the drawback that infected mice do not develop necrotizing granulomas. These structures are the hallmark for disease in human TB and are believed to play a central role in mycobacterial persistence (62, 63). For the experiments described in this thesis we use the BALB/c mouse model, because, despite the absence of necrotizing granulomas, the course of infection and treatment in the BALB/c mice resembles the clinical situation remarkably (64). After several months of treatment no mycobacteria can be cultured from the lungs, but a full 6-months course with the current TB drug regime is necessary to eradicate persistent mycobacteria and prevent relapse of disease (64). Eradicating these persistent mycobacteria more efficiently is the key to shortening treatment duration and their proven presence in the BALB/c mouse model indicates its usefulness as preclinical model.

## OUTLINE OF THIS THESIS

The aim of this thesis is to increase our understanding of TB pathogenesis and improve its treatment. Therefore, mycobacterial-, host-, and treatment factors are studied.

### Mycobacterial factors

To what degree does mycobacterial strain diversity influences treatment outcome and host responses in mouse TB models? The mycobacterial strain H37Rv is still commonly used in preclinical TB research, but it is more than 100 years old and no longer isolated from patients, can we therefore still use it as model organism? In **Chapter 3** we analyze host-responses against H37Rv compared to two recently isolated clinical strains from the Beijing and East-African Indian genotype to evaluate how currently circulating strains evade protective immunity more efficiently. To evaluate the impact of strain diversity on TB treatment, we assess bactericidal drug activity and treatment outcome against recent clinical isolates in **Chapter 5**.

### Host factors

Both impaired host responses and boosting immunity can result in disease progression in TB, indicating the duality and importance of our immune system in TB pathogenesis. In the current paradigm, IL-12 stimulates IFN-γ-mediated macrophage activation and mycobacterial killing, which is essential in TB as observed in patients with MSMD. How-

ever, this does not explain the recently identified functional role of antibodies and B-cells in TB (65, 66). Also the emerging s of type 1 interferons and/or Th17 immunity in patients need to be incorporated into our current understanding of TB pathogenesis. Therefore we have performed a review of the current literature on these factors in **Chapter 2**. We also evaluate the feasibility of altering host responses through host-directed therapy adjunct to antibiotic treatment to improve treatment outcome in **Chapter 4.**

**Treatment factors**

Poor outcomes of recent clinical phase III trials evaluating novel TB treatment regimens have shown that the predictive value of preclinical models needs to be further optimized (58). In **Chapter 5** we validate the efficacy of conventional TB drugs in our own mouse TB model using a mycobacterial Beijing genotype strain and assess the predictive value of early bactericidal activity, i.e. during the first months of treatment, on treatment outcome. Finally, in **Chapter 6** we present a new approach for treatment outcome evaluation by combining observational data with mathematical modeling in order to evaluate the potency of (new) TB drug regimens.

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